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Inactivation of Membrane Tumor Necrosis Factor Alpha by Gingipains from *Porphyromonas gingivalis*

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Gingipains are cysteine proteinases produced by *Porphyromonas gingivalis*, a major causative bacterium of adult periodontitis. They consist of arginine-specific (HRgpA and RgpB) and lysine-specific (Kgp) proteinases. Gingipains strongly affect the host defense system by degrading some cytokines, components of the complement system, and several immune cell receptors. In an in vitro model, gingipains were shown to degrade soluble tumor necrosis factor alpha (TNF-α). However, since membrane TNF-α shows strong biological activity, especially in local inflammatory lesions, it was worth investigating whether gingipains might also destroy membrane TNF-α and limit its biological activities. To avoid a possible influence of gingipains on ADAM17, the secretase of TNF-α, the majority of experiments were performed using ADAM17$^{-/-}$ fibroblasts stably transfected with cDNA of human pro-TNF-α (ADAM17$^{-/-}$ TNF^{+}). Arginine-specific gingipains (Rgp's) strongly diminished the level of TNF-α on the cell surface as measured by flow cytometry, and this process was not accompanied by an increased concentration of soluble TNF-α in the culture medium. Degradation of membrane TNF-α by Rgp's correlated with a strong decrease in TNF-α-mediated biological activities of ADAM17$^{-/-}$ TNF^{+} cells. First, the activation state of transcription factor NF-κB was suppressed; second, the cells were no longer able to induce apoptosis in HL-60 cells. Kgp was also able to cleave membrane TNF-α, but its effect was much weaker than that of Rgp's. Gingipains also limited the binding of native TNF-α to the target cells. Thus, gingipains are able not only to cleave soluble TNF-α but also to destroy the membrane form of the cytokine, which may additionally dysregulate the cytokine network.

Adult periodontitis is the most prevalent form of periodontal disease and results from chronic inflammatory processes initiated in the gingiva and extending to the supporting periodontal tissues (31). The disease is characterized by massive accumulation of immune cells (neutrophils, dendritic cells, T cells), bone resorption, formation of periodontal pockets, and loss of tooth attachment (29, 31). Severe forms of periodontitis may result not only in tooth loss but also in systemic complications such as cardiovascular diseases (18) or preterm delivery (10).

Porphyromonas gingivalis, a gram-negative, anaerobic, asaccharolytic bacterium, has been implicated as a major causative pathogen in the initiation and progression of adult periodontitis. A variety of virulence factors, including lipopolysaccharide (LPS), fimbriae, proteinases, and hemagglutinins, enable the bacterium to colonize periodontal pockets and counteract host defense mechanisms (31). Gingipains, cysteine proteinases that comprise the vast majority of *P. gingivalis* proteolytic activity, appear to contribute significantly to the pathogenesis of periodontitis by degrading constituents of periodontal tissue; dysregulating coagulation, complement, and kallikrein-kinin cascades; and destroying cytokines, cell adhesion molecules, and immune cell receptors including CD14, CD4, and CD8 (30, 39, 49). These deleterious effects of gingipains can be manifested in vivo, because the enzymes are resistant to inhibition by human plasma and tissue proteinase inhibitors (11).

The two types of gingipains are referred to as arginine specific (HRgpA and RgpB) and lysine specific (Kgp) because of their specificity for cleavage after arginyl and lysyl residues, respectively. Although HRgpA and RgpB are products of two distinct genes, they have nearly identical catalytic subdomains; a major difference is the absence of the hemagglutinin domain in RgpB (13).

All three members of the gingipain family have been shown to degrade, although with different efficiencies, the major proinflammatory cytokine soluble tumor necrosis factor alpha (TNF-α) (9). Molecules synthesized in response to TNF-α, such as cell adhesion receptors, interleukin 8 (IL-8), IL-6, major histocompatibility complex class I, and nitric oxide, constitute key components of the host's antibacterial warfare. Thus, degradation of TNF-α by gingipains may be one mechanism by which *P. gingivalis* evades the host defense system.

Overproduction of TNF-α can have deleterious effects contributing to acute and chronic inflammatory diseases. TNF-α, in conjunction with IL-1, appears to be responsible for connective tissue destruction and bone resorption during the progression of periodontitis, since antagonists to IL-1 and TNF-α significantly limit tissue damage in experimental periodontitis (20). We hypothesized that since soluble TNF-α is efficiently degraded by gingipains, the deleterious effects observed might be due to membrane TNF-α activity.

Here we present the results of our studies on the effects of

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gingipains on membrane TNF-α and discuss possible consequences of those effects.

MATERIALS AND METHODS

Cell lines. ADAM17$^{-/-}$ Ras-Myc-immortalized murine fibroblasts isolated from ADAM17$^{\Delta Zn/\Delta Zn}$ mice were provided by Amgen, Inc. (Seattle, Wash.). The other cell lines, including the human histiocytic lymphoma cell line U937 (47) and the acute promyelocytic leukemia cell line HL-60 (ATCC CCL-240) were purchased from the American Type Culture Collection.

pcDNA3.1zeo/proTNFα, containing the entire coding sequence of human TNF-α (huTNF-α) cDNA, was a generous gift form Stefan Rose-John (Mainz, Germany). Oligonucleotide probes were synthesized in the Institute of Biochemistry and Biophysics PAN (Warsaw, Poland). Horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG), fluorescein isothiocyanate-conjugated goat anti-mouse IgG, and goat anti-huTNF-α IgG were from Sigma Chemical Co. (St. Louis, Mo.). MitoTracker Red CMXRos was from Molecular Probes (Eugene, Oreg.), while all tissue culture reagents, including Dulbecco's modified Eagle medium (DMEM)–Glutamax-1, fetal bovine serum, and trypsin-EDTA, were purchased from Gibco BRL/Life Technologies (Paisley, United Kingdom). The proteinase inhibitors benzyloxycarbonyl-Phe-Lys-CH_2OCP-2,4,6-Me3-Ph (Z-FK-ck) and Phe-Pro-Arg-chloromethylketone (FPR-ck) were from Bachem Biosciences, Philadelphia, Pa., and leupeptin was from Sigma.

Generation of ADAM17$^{-/-}$ cells stably transfected with pro-TNF-α cDNA (ADAM17$^{-/-}$ TNF^{+} cell line). ADAM17$^{-/-}$ cells were transfected with pcDNA3.1zeo/proTNFα by using the calcium phosphate transfection method in an *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanasulfonic acid-buffered system (5). After selection on zeocin (500 μg/ml), the clones expressing high levels of membrane TNF-α were identified by flow cytometric analysis and further propagated. Cell cultures that contained between 65 and 90% TNF-α-positive cells were used for experiments.

Cell cultures. All cell lines were cultured in DMEM–Glutamax-1 supplemented with 10% fetal bovine serum (complete medium). Culture media for ADAM17$^{-/-}$ and ADAM17$^{-/-}$ TNF^{+} cells were enriched in Geneticin (G418; 1.5 mg/ml), and the medium for ADAM17$^{-/-}$ TNF^{+} cells was additionally enriched in zeocin (500 μg/ml), every few passages. Cell cultures were maintained at 37°C under 5% CO_2 and were passaged by trypsinization after reaching 80 to 90% confluence.

Purification and activation of gingipains. RgpB, HRgpA, and Kgp were purified from *P. gingivalis* culture as described previously (38, 40), and their purity was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The amounts of active gingipains were estimated by using active-site titration with the inhibitor Z-FK-ck or FPR-ck for lysine or arginine gingipains, respectively (41), and the concentrations of gingipains indicated in this study represent those of active enzymes.

Gingipains were activated by incubation of the enzymes in 0.2 mM HEPES supplemented with 1 mM $CaCl_2$ and 20 mM cysteine (pH 8.0) at 37°C for 10 min. After incubation, gingipains were diluted in DMEM supplemented with 10 mM cysteine to the desired concentrations and were immediately used for experiments.

Analysis of gingipain-mediated TNF-α shedding. After trypsinization, ADAM17$^{-/-}$ TNF^{+} cells were suspended in DMEM supplemented with 10 mM cysteine and placed in round-bottom 96-well plates (4×10^5 cells/well). The cells were incubated for 5, 15, or 50 min (unless indicated otherwise) with various concentrations of activated gingipains. A gingipain inhibitor (Z-FK-ck [0.4 μM] or leupeptin [100 μM]) or the protein synthesis inhibitor cycloheximide (5 μg/ml) was added to some wells prior to the addition of gingipains. After incubation, the enzymatic activity of Kgp was terminated by addition of Z-FK-ck (0.4 μM), and those of RgpB and HRgpA were terminated by addition of leupeptin (100 μM). Then the cells were subjected to flow cytometric analysis, and supernatants were analyzed for the presence of the TNF-α antigen by enzyme-linked immunosorbent assays (ELISA) and Western blotting.

Monocytes isolated from the blood of healthy donors by counterflow centrifugation elutriation were incubated for 16 h in a V-bottom 96-well plate (10^5 cells/well) in the presence of LPS (500 ng/ml) to stimulate TNF-α synthesis. Then the cells were incubated for 50 min with different concentrations of activated gingipains. Surface TNF-α levels were estimated by using flow cytometric analysis.

Analysis of the effects of gingipains on TNF-α binding to the cell surface. Undifferentiated U937 cells were incubated for 15 min at 37°C in 96-well plates (10^5 cells per well) in a medium supplemented with 10 mM cysteine in the absence or the presence of activated gingipains at 10, 100, or 300 nM. The reaction was stopped by inhibiting the activity of Kgp with Z-FK-ck (0.5 μM) and those of Rgp's with leupeptin (100 μM). The ability of the cells to bind TNF-α was assayed as described by Bu et al. (8). Briefly, after blocking of gingipain activity, U937 cells were incubated with TNF-α (200 ng/ml) for 30 min on ice. Then the cells were washed five times with ice-cold phosphate-buffered saline (PBS) supplemented with 2% fetal calf serum and 0.02% NaN_3, and the levels of TNF-α bound to the cells were examined by flow cytometry. U937 cells that were not treated with TNF-α were used as a control for staining specificity.

Flow cytometric analysis. ADAM17$^{-/-}$ TNF^{+} cells, monocytes, or U937 cells were incubated for 1 h on ice in complete medium containing an anti-huTNF-α monoclonal antibody (MAb) (10 μg/ml) (murine, generated in our laboratory), washed with PBS, and then incubated for 30 min on ice in complete medium containing fluorescein isothiocyanate-conjugated anti-murine IgG (20 μg/ml). For U937 cells, all incubations were carried out in the presence of 0.029. NaN_3 to prevent internalization of the bound TNF-α. The staining of the cells versus that of isotype controls was analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, Calif.) using CellQuest software.

ELISA. TNF-α levels in culture media of ADAM17$^{-/-}$ TNF^{+} cells were measured by using a sandwich ELISA (37). After incubation of the cells with gingipains, the media were collected and diluted (30-fold), and 100-μl samples were applied to Maxisorp ELISA plates (Nalge Nunc Inc., Roskilde, Denmark) coated with polyclonal anti-huTNF-α IgG. Plates were incubated for 2 h at room temperature and, after unbound proteins were washed out, were incubated first with a murine anti-huTNF-α MAb (0.2 μg/ml in PBS–0.5% bovine serum albumin [BSA]) for 1 h at room temperature and then with HRP-conjugated anti-murine IgG (0.1 μg/ml in PBS–0.5% BSA) for 30 min at room temperature. Each incubation step was followed by intensive washing of the plate with PBS. The enzymatic reaction was performed by using the TMB substrate reagent set (PharMingen, San Diego, Calif.), and absorbance was measured at a λ of 450 nm.

Western blot analysis. Samples of media (20 μl) collected after incubation of ADAM17$^{-/-}$ TNF^{+} cells with gingipains were separated in a 15% polyacrylamide gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene difluoride membrane (Millipore, Billerica, Mass.), and Western blot staining was performed as described in reference 12, with modifications. Briefly, the membranes were incubated for 1 h in the blocking buffer (2% BSA in Tris-buffered saline–0.1% Tween 20) followed by the primary antibodies (murine anti-TNF-α IgG) at a concentration of 2 μg/ml and then the secondary antibody (HRP-conjugated rabbit IgG against murine IgG) at a concentration of 0.05 μg/ml. Both the primary and secondary antibodies were diluted in the blocking buffer, and Tris-buffered saline was used for washing the membranes before, between, and after their incubations with antibody solutions. Antibody-reactive proteins were detected by using enhanced chemiluminescence reagents (ECL kit; Amersham Pharmacia Biotech, Uppsala, Sweden).

EMSA. Inhibition of TNF-α-mediated NF-κB activation by gingipains was analyzed by using electrophoretic mobility shift assays (EMSA). A total of 10^6 ADAM17$^{-/-}$ TNF^{+} cells (used as both TNF-α-expressing cells and TNF-α-responders) were incubated for 1 h in the absence or presence of a particular gingipain at various concentrations. Then nuclear extracts were isolated by the method of Suzuki et al. (48). Nuclear proteins were suspended in EB buffer (0.35 M NaCl, 5 mM EDTA, 1 mM dithiothreitol, 10 mM Na-HEPES [pH 7.5], 0.2 mM phenylmethylsulfonyl fluoride) supplemented with 10% glycerol. An oligonucleotide probe containing the κB site from the mouse κ-light chain enhancer (5′-AGCTTCAGAGGGGACTTTCCGAGAGG-3′ and 5′-AGCTCCTCTCGG AAAGTCCCCTCTGA-3′) was labeled with [α-^{32}P]dCTP by using the Klenow fragment of DNA polymerase I (Fermentas, Vilnius, Lithuania). Labeled probe was separated from unincorporated nucleotides by use of the QIAquick nucleotide removal kit (QIAGEN GmbH, Hilden, Germany). Nuclear extracts (6 μg per assay) were incubated with 0.5 to 2 ng (2×10^4 cpm) of the probe and 1.5 μg of poly(dI-dC) for 30 min at room temperature in a total reaction mixture of 25 μl containing 2 mM Na-HEPES (pH 7.5), 0.1%Triton X-100, 0.5% glycerol, and 1 mM dithiothreitol. Protein-DNA complexes were separated in a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA. The gels were vacuum dried and exposed to a phosphorimager screen. The autoradiograms were analyzed by using Personal Molecular Imager FX (Bio-Rad, Hercules, Calif.) and Quantity One software (Bio-Rad).

Analysis of mitochondrial membrane potential in HL-60 cells. HL-60 cells were preincubated for 20 min with cycloheximide (250 ng/ml). At the same time ADAM17$^{-/-}$ TNF^{+} and ADAM17$^{-/-}$ cells plated in a 96-well plate (2.5×10^5/well) were pretreated with particular gingipains at 300 nM. Then the HL-60 cells (7.5×10^4) were coincubated with the ADAM17$^{-/-}$ TNF^{+} or ADAM17$^{-/-}$ cells in the presence of 300 nM gingipains and cycloheximide (250 ng/ml) for 3 h. Every 50 min, the medium was replaced with fresh medium containing new portions of gingipains and cycloheximide. The magnitude of TNF-α-induced apoptosis of HL-60 cells was measured by evaluation of mitochondrial membrane

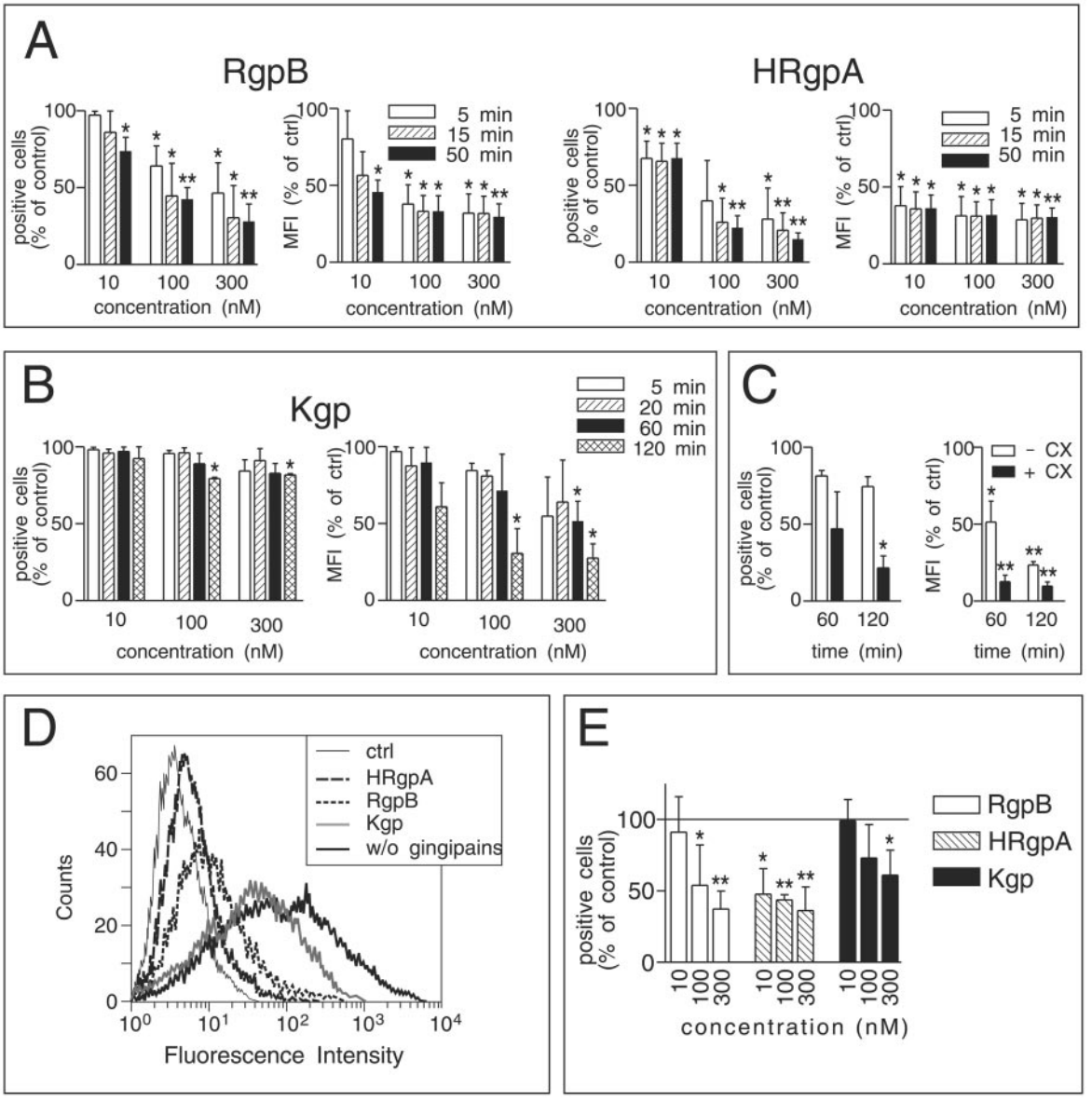

FIG. 1. Flow cytometric analysis of TNF-α levels on the cell surface. (A to C) TNF-α levels on the surfaces of ADAM17$^{-/-}$ TNF^{+} cells incubated with HRgpA or RgpB (A), Kgp (B), or Kgp in the presence of cycloheximide (C). (D) Histogram comparing the effects of all three gingipains on TNF-α levels on the surfaces of ADAM17$^{-/-}$ TNF^{+} cells. (E) TNF-α levels on the surfaces of human peripheral blood monocytes incubated with different gingipains. Data represent arithmetic-mean (average) percentages of cells that are TNF-α positive and average mean fluorescence intensities (MFI) of the TNF-α-positive cells ± standard deviations from four independent experiments for panels A, B, and E and from two independent experiments for panel C. The percentage of cells that were TNF-α positive and the MFI of control (non-gingipain-treated) cells were taken as 100%. *, $P < 0.05$ versus control cells; **, $P < 0.01$ versus control cells.

potential. Cell mixtures were incubated for 15 min at 37°C in a medium supplemented with MitoTracker Red CMXRos (200 nM), whose mitochondrial accumulation and thus fluorescence intensity depends on membrane potential. After incubation, the cells were analyzed by flow cytometry with an acquisition gate set on the easily distinguishable HL-60 population, defined by forward scatter and side sctter signals. The analysis was performed using the CellQuest program.

Statistical analysis. Statistical analysis was performed using the Student *t* test. A *P* value of <0.05 was considered significant.

RESULTS

Arginine-specific gingipains efficiently cleave membrane TNF-α. We examined the abilities of HRgpA, RgpB, and Kgp to cleave the membrane form of TNF-α. To avoid a possible influence of gingipains on ADAM17, the secretase responsible for TNF-α shedding, the majority of experiments were performed using ADAM17-deficient fibroblasts stably transfected with human pro-TNF-α cDNA (ADAM17$^{-/-}$ TNF^{+} cells).

The concentration of gingipains in the gingival crevicular fluid (GCF) from periodontitis patients is about 100 nM. Since the enzymes are present not only in the soluble form but also bound to the bacterial surface and are included into outer membrane blebs, the local concentration of gingipains might be significantly higher (46). In this study we used gingipains at

TABLE 1. Reexpression of TNF-α on the cell surface after removal of gingipains

Gingipain	Before gingipain treatment		After gingipain treatment		1 h after gingipain removal	
	% TNF^+	MFI	% TNF^+	MFI	% TNF^+	MFI
RgpB	85	327	43	30	84	325
HRgpA	85	327	20	27	86	315

the concentrations of 10, 100, and 300 nM as probably the most relevant to in vivo conditions.

As determined by flow cytometric analysis, incubation of the ADAM17$^{-/-}$ TNF^+ cells with either type of gingipain resulted in time- and dose-dependent decreases in TNF-α levels on the cell surface as well as in the number of TNF-α-positive cells. HRgpA showed the most potent proteolytic activity toward membrane TNF-α (Fig. 1A and D). When the cells were treated with 300 or 100 nM HRgpA, the binding of the anti-TNF-α MAb to the cell surface was strongly decreased after a 5-min incubation of the cells with the enzyme. The effect of 10 nM HRgpA was less pronounced but still significant. Degradation of membrane TNF-α by RgpB was also substantial, although the process was not as fast and effective as that caused by corresponding concentrations of HRgpA (Fig. 1A and D). In contrast to arginine-specific gingipains, Kgp did not efficiently cleave membrane TNF-α. A moderate decrease in TNF-α levels on ADAM17$^{-/-}$ TNF^+ cells, as judged by fluorescence intensity, was observed only after incubation of the cells with 300 and 100 nM Kgp for prolonged periods (Fig. 1B and D).

In the case of slow cleavage of membrane TNF-α by a protease, it is possible that the loss of the cell surface cytokine can be compensated for by de novo synthesis and surface expression of TNF-α. This process could be responsible for the apparent ineffectiveness of TNF-α degradation by Kgp. To test this hypothesis, levels of TNF-α were measured on cells preincubated with cycloheximide for 1 h and then incubated with 300 nM Kgp in the presence of cycloheximide for an additional 1 or 2 h. When reexpression of TNF-α was prevented, Kgp strongly decreased surface TNF-α levels, indicating that the enzyme was able to remove TNF-α from the cell membrane (Fig. 1C).

The presence of specific protease inhibitors during the incubation of the cells with gingipains prevented the release of TNF-α from the cell membrane, indicating that this effect was fully dependent on the proteolytic activity of gingipains (data not shown).

The observed decrease in the TNF-α level did not result from any deleterious effect of gingipains on the cells, since cell viability did not change upon incubation with gingipains, as estimated by trypan blue exclusion. Accordingly, a 1-h incubation of the cells in fresh medium after removal of gingipains led to complete restoration of membrane TNF-α levels (Table 1).

Since ADAM17$^{-/-}$ TNF^+ cells are not physiological TNF-α producers, we also examined the effects of gingipains on membrane TNF-α by using a more physiological cell model, human peripheral blood monocytes. We found that particular gingipains decreased the TNF-α level on the monocyte surface with an effectiveness similar to that observed for the ADAM17$^{-/-}$ TNF^+ cells (Fig. 1E).

Arginine-specific gingipains efficiently degrade soluble TNF-α. Next, we examined the levels of TNF-α in the culture medium of the gingipain-treated ADAM17$^{-/-}$ TNF^+ cells in order to estimate whether soluble TNF-α removed from the membrane accumulates or is immediately degraded by the proteases. Although our model cells are deficient in ADAM17, the major TNF-α secretase, release of TNF-α was not completely abolished, possibly due to the activity of ADAM10 and/or MMP7. Thus, TNF-α was always present at basal levels in the culture medium of control (non-gingipain-treated) cells.

When the cells were incubated with arginine-specific gingipains, we observed no increase, but rather a significant decrease, in the basal concentration of TNF-α in the culture medium (Fig. 2). This may indicate either that gingipains immediately digested the solubilized cytokine or that they degraded the ectodomain of TNF-α directly on the cell surface. The decrease in the TNF-α level in the medium of RgpB-treated cells was more dramatic than that observed for cells subjected to HRgpA treatment. Incubation of cells with 10 nM RgpB resulted in a decrease of about 50% in the basal TNF-α level, and only trace amounts of the cytokine were detected in the presence of 300 nM RgpB. A significant effect of RgpB was already visible after 5 min of incubation. In contrast, about 25% of the basal TNF-α level was still detectable in the medium of the cells treated for 50 min with 300 nM HRgpA. Thus, HRgpA shows higher proteolytic activity than RgpB toward membrane TNF-α, but RgpB is more potent than HRgpA in degradation of the soluble cytokine. These results were further confirmed by Western blot analysis (Fig. 2C). Lower concentrations of RgpB than of HRgpA were required to decrease the 17-kDa band corresponding to the soluble form of TNF-α after incubation with the cells for 50 min.

Soluble TNF-α, like the membrane form of TNF-α, was much more resistant to Kgp than to arginine-specific gingipains. A slight decrease in the TNF-α level in the culture medium could be observed only after treatment of cells with high doses of Kgp (300 nM) for 20 and 50 min (Fig. 2D).

Gingipains inhibit biological activities of membrane TNF-α. Although gingipains are able to destroy membrane TNF-α and to degrade the soluble cytokine efficiently, the question arises whether the residual level of the functional cytokine can still exert biological activity. To address this question, we measured the activation of transcription factor NF-κB, which is known to be constitutively activated in cells stably transfected with TNF-α and expressing TNF receptors (24), apparently due to the autocrine action of TNF-α. In our experiments we examined the effects of gingipains on the state of NF-κB activation in ADAM17$^{-/-}$ TNF^+ cells (the level of NF-κB activation in ADAM17$^{-/-}$ cells that were not transfected with TNF-α was negligible). After incubation of the cells with either HRgpA, RgpB, or Kgp for 1 h, the level of activated NF-κB in nuclear extracts was determined by an EMSA. All three gingipains were able to diminish the level of activated NF-κB in ADAM17$^{-/-}$ TNF^+ cells. HRgpA exerted the strongest effect, and Kgp had the weakest (although still significant) (Fig. 3).

We also examined whether gingipains are able to inhibit apoptosis of HL-60 cells induced by coincubation of the TNF-α-sensitive HL-60 cells with ADAM17$^{-/-}$ TNF^+ cells. Initia-

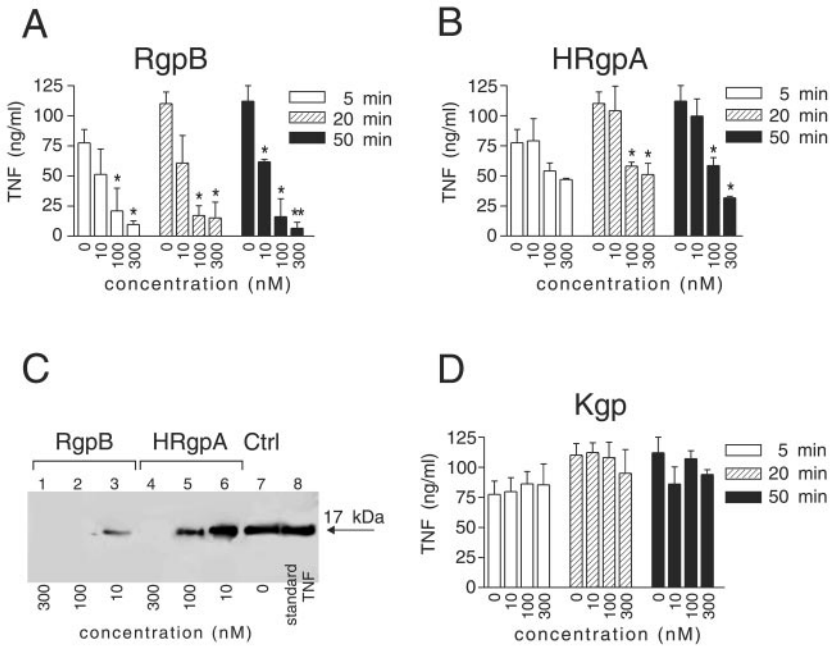

FIG. 2. Analysis of soluble TNF-α levels. Levels of TNF-α in the culture media of ADAM17$^{-/-}$ TNF^{+} cells incubated with RgpB (A), HRgpA (B), RgpB or HRgpA (C), or Kgp (D) were analyzed by ELISA (A, B, and D) or by Western blotting (C). Ctrl, control. Data in panels A, B, and D are shown as averages ± standard deviations for three experiments performed. *, $P < 0.05$ versus control cells; **, $P < 0.01$ versus control cells.

tion of apoptosis was evaluated by flow cytometric analysis of depolarization of the mitochondrial membrane in HL-60 cells labeled with the specific fluorescent probe MitoTracker Red CMXRos. As shown in Fig. 4A, incubation of HL-60 cells with ADAM17$^{-/-}$ TNF^{+} cells resulted in a significant decrease in red fluorescence intensity, which correlates with mitochondrial membrane potential. In contrast, when HL-60 cells were coincubated with the original ADAM17$^{-/-}$ cells, which do not express TNF-α, only a slight decrease in mitochondrial membrane potential was observed. This result indicates that mainly TNF-α, and not other factors expressed by ADAM17$^{-/-}$ cells, was responsible for ADAM17$^{-/-}$ TNF^{+}-mediated apoptosis of HL-60 cells (Fig. 4). Pretreatment of ADAM17$^{-/-}$ TNF^{+} cells with HRgpA and the presence of HRgpA in the medium during coincubation of HL-60 cells with ADAM17$^{-/-}$ TNF^{+} cells strongly suppressed TNF-α-induced depolarization of the mitochondrial membrane. RgpB also inhibited TNF-α-induced apoptosis, although its effect was less pronounced than that of HRgpA. Kgp had only a minor effect on this process (Fig. 4B).

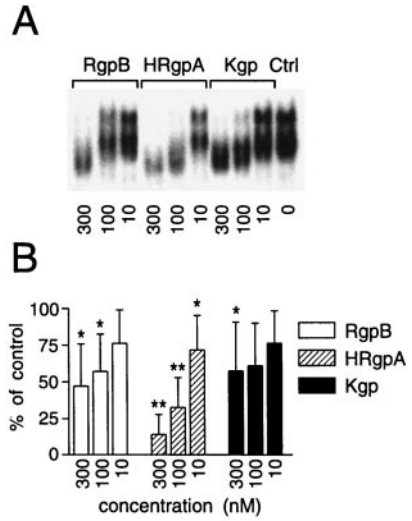

FIG. 3. EMSA analysis of the effects of gingipains on NF-κB activation. ADAM17$^{-/-}$ TNF^{+} cells were incubated with gingipains for 1 h, and nuclear extracts were subjected to EMSA with the NF-κB-specific probe. (A) A single autoradiogram representative of five experiments. (B) Bars represent average autoradiography signals ± standard deviations for NF-κB obtained by EMSA analysis of five independent experiments. *, $P < 0.05$ versus control cells; **, $P < 0.01$ versus control cells.

Gingipains inhibit binding of TNF-α to target cells. The observation that Kgp, which only slightly affects the level of membrane TNF-α and is ineffective at degradation of the soluble cytokine, is able to moderately inhibit TNF-α-mediated activation of NF-κB prompted us to investigate whether gingipains may degrade TNF receptors.

Undifferentiated U937 cells do not produce TNF-α but are responsive to this cytokine. Therefore, the amount of exogenous TNF-α bound to U937 cells and determined by flow cytometry can be considered an indirect measure of the surface levels of TNF receptors (8). U937 cells were incubated for 15 min with gingipains at different concentrations, and after blocking of the enzymatic activity, the ability of the cells to bind TNF-α was examined.

The most potent decrease in TNF-α binding was observed after treatment of U937 cells with HRgpA (Fig. 5). The difference in effectiveness between HRgpA and RgpB was signif-

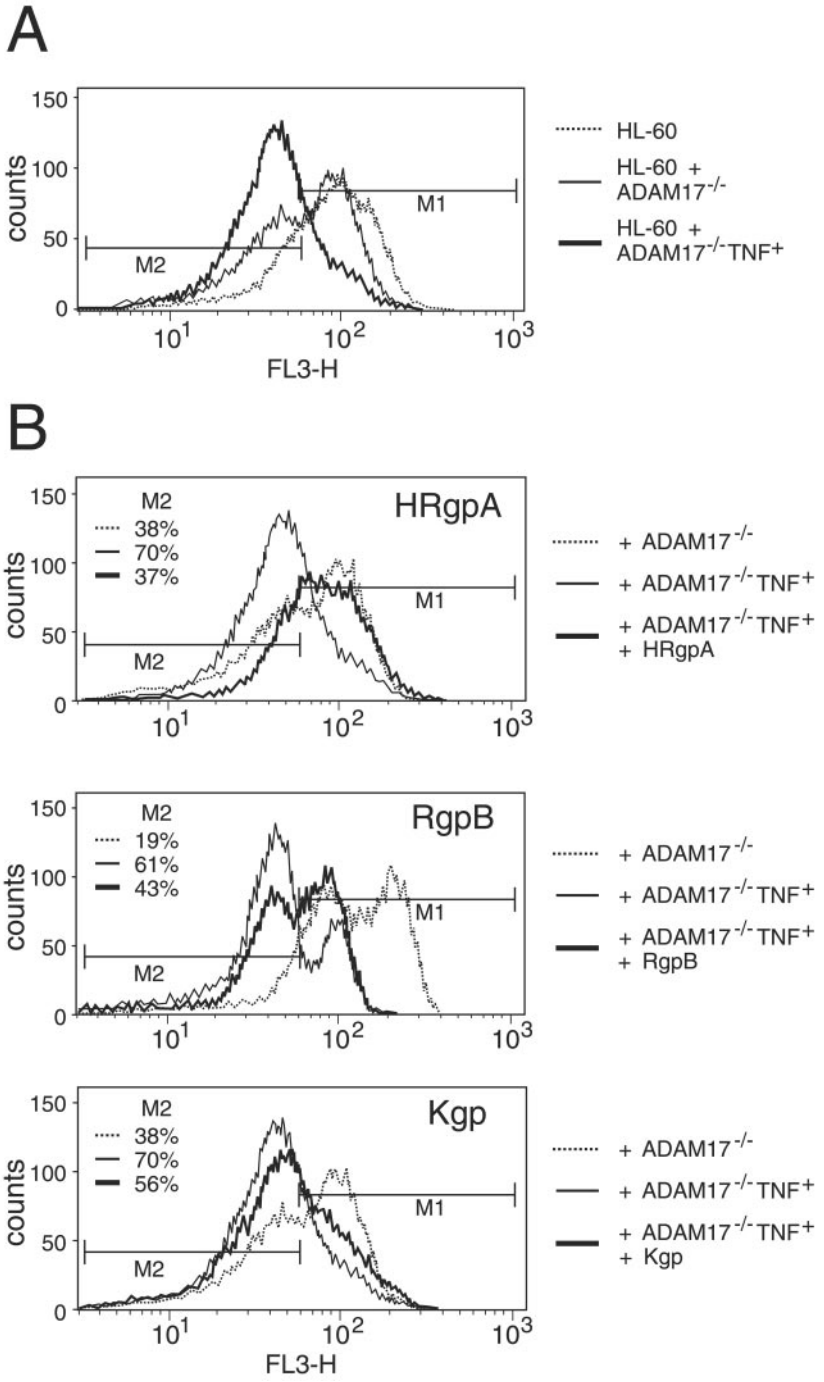

FIG. 4. Flow cytometric analysis of MitoTracker Red CMXRos incorporation into HL-60 cells coincubated with ADAM17$^{-/-}$ or ADAM17$^{-/-}$ TNF$^+$ cells (A) or ADAM17$^{-/-}$ TNF$^+$ cells in the absence or presence of gingipains (B). The acquisition gate was set on the HL-60 population defined by FSC/SSC signals. For the analysis the M1 gate was set on the cells showing high fluorescence intensity and the M2 gate was set on the cells showing low fluorescence intensity (apoptotic). Numbers in charts represent the percentages of cells viewed as apoptotic. Data are from a single experiment representative of two performed.

icant only when these gingipains were compared at the lowest enzyme concentration studied (10 nM). Kgp at concentrations of 10 and 100 nM affected the binding of TNF-α to U937 cells to a lesser extent than arginine-specific gingipains, but at 300 nM the effects of all three gingipains were comparable (Fig. 5). Thus, Kgp may influence the biological effects of TNF-α by affecting its interaction with target cells. The potential of gingipains to limit TNF-α binding to cells probably results from their ability to hydrolyze TNF receptors. This hypothesis needs to be confirmed in further studies in which the levels of TNFR I and TNFR II in control and gingipain-treated cells will be examined directly by using antibodies recognizing individual receptors.

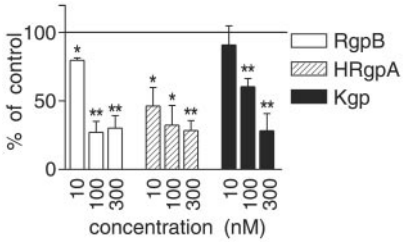

FIG. 5. Analysis of TNF-α binding to gingipain-treated U937 cells. U937 cells were incubated with gingipains and then, after inhibition of enzymatic activity, with TNF-α. The levels of bound TNF-α were estimated by flow cytometry. Bars represent the average percentages of cells that were stained ± standard deviations from three independent experiments. *, $P < 0.05$ versus control cells; **, $P < 0.01$ versus control cells.

DISCUSSION

TNF-α is a major proinflammatory cytokine with broad immune effects (3). This pleiotropic cytokine is expressed mainly by macrophages as a 26-kDa transmembrane protein that is converted to a 17-kDa soluble molecule by a membrane bound secretase, ADAM17 (7). Both soluble and transmembrane TNF-α are biologically active. It has been demonstrated that membrane TNF-α, like its soluble counterpart, is able to induce apoptosis in various cell types (17, 23, 32, 35), to activate T cells and stimulate thymocyte proliferation (22), and to trigger expression of VCAM-1 and tissue factor in endothelial cells (35, 43). However, the overall biological consequences of the actions of the two forms are not always identical, primarily because only soluble TNF-α may exert systemic effects (35). There are two TNF-α receptors, which direct signal transduction pathways that only partially overlap (26, 33). Both soluble and membrane TNF-α may interact with both receptors, although membrane-bound rather than soluble TNF-α is the prime physiological activator of TNFR II (22). TNFR I is expressed in all cell types, whereas only immune and endothelial cells express TNFR II (2). It has been shown that membrane TNF-α induces cooperative signaling of both receptors and thus in some instances may elicit stronger effects than soluble TNF-α (32, 51).

As a proinflammatory cytokine, TNF-α causes phenotypic changes in a number of cells, and the activity of molecules synthesized in response to TNF-α constitutes an important element of defense against pathogens and injuries (3). However, prolonged activation of cells in response to TNF-α may have deleterious effects contributing to chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease, glomerulonephritis, systemic lupus erythematosus, and others (1).

Adult periodontitis is classified as a chronic inflammatory disease, and the majority of tissue damage caused by periodontal pathogens may be attributed to deleterious effects of inflammatory mediators such as IL-1 and TNF-α rather than to a direct influence of the bacteria and bacterial products (21). However, the role of individual cytokines, especially TNF-α, in the pathology of this disease is controversial. There is no doubt that TNF-α exerts several biological activities that are highly related to pathological changes in the inflamed periodontal tissue. This cytokine stimulates fibroblast apoptosis, increases vascular permeability, and contributes to recruitment of polymorphonuclear leukocytes (PMN) (21). Furthermore, it stimulates the differentiation and activation of osteoclasts, involved in bone resorption (6, 42), and induces the synthesis of matrix metalloproteases (MMP1, MMP2, MMP9) in fibroblasts, macrophages, and osteoclasts. Taken together, these activities could theoretically contribute to pathological changes associated with periodontitis manifested in such clinical hallmarks of the disease as PMN accumulation, formation of GCF, and loss of tooth attachment due to excessive destruction of connective tissue and bone resorption (28, 36, 50). Indeed, an association between the severity of periodontitis and single-nucleotide polymorphisms in the TNF-α promoter, related to high TNF-α inducibility, has been demonstrated (44). In addition, a correlation between the clinical parameters of periodontal lesions and levels of TNF-α in gingival tissue has been shown (19). Despite these observations, numerous reports undermine the significance of TNF-α involvement in the progression of periodontitis (9, 16, 46) and implicate IL-1 as the major factor responsible for the clinical outcome of this disease (14, 45, 52). The observation that IL-1β is present at significantly higher levels than TNF-α in GCF and inflamed periodontal tissue additionally supports this hypothesis (15, 19, 45, 52).

The discrepancy in views on the role of TNF-α in periodontitis may be due to two factors frequently overlooked by investigators. First, a variety of cytokines, often with overlapping activities, work in concert at sites of inflammation, and it is usually impossible to attribute a given effect to a single cytokine. Second, the host-derived and/or bacterial proteases in inflamed periodontal tissue or GCF can be responsible for local elimination of an individual cytokine and/or its signaling pathway(s) or even the receptors needed for the induction of a given cytokine.

Indeed, gingipains, both soluble and associated with outer membrane vesicles, released in large amounts by *P. gingivalis*, have been shown to shed and degrade the LPS receptor, CD14, which may result in diminished inducibility of TNF-α (16, 46). However, the synthesis of TNF-α is not completely abolished (34), possibly due to other stimulatory pathways (25). Nevertheless, the activity of TNF-α in inflamed periodontal tissue should be attenuated, since released TNF-α is hydrolyzed by gingipains (9). If so, it could be hypothesized that the deleterious effects are attributable to the membrane form of TNF-α. However, we have rejected this hypothesis. In the present work we have demonstrated that gingipains are able to degrade not only soluble but also membrane TNF-α with comparable efficacy (Fig. 1 and 2).

The arginine-specific enzymes were more efficient than Kgp in degrading both forms of TNF-α. When the activities of HRgpA and RgpB were compared, the first enzyme was more potent toward membrane TNF-α and the second was more potent toward soluble TNF-α. The higher efficiency of proteolysis of membrane TNF-α by HRgpA may result from the presence of the adhesin subunit that facilitates interactions of the enzyme with the cell membrane proteins, thus increasing the probability of enzyme-substrate encounter. However, the possibility that the catalytic domains of the two gingipains differently recognize the peptide bonds Arg60–Xaa61 and/or Arg65–Xaa66, present only in membrane and not in soluble TNF-α, cannot be excluded. The importance of differences in

the active sites between HRgpA and RgpB for their different substrate specificities has been demonstrated recently (4).

Degradation of membrane TNF-α by gingipains is not accompanied by accumulation of the cytokine in the culture medium. Two scenarios are possible. Gingipains may gradually hydrolyze several susceptible bonds present in soluble as well as in membrane TNF-α molecules. It is also possible that some TNF-α molecules are cleaved within the 20-amino-acid fragment between the cell surface and the ADAM17-susceptible bond (Ala76:Val77) or in its close vicinity. This fragment of human TNF-α contains several Arg residues (Arg60, -65, -78, and -82) that could be targets for gingipains. If this situation occurs, shedding of TNF-α is immediately followed by degradation of the soluble molecule, which prevents accumulation of TNF-α in the culture medium.

We have also demonstrated that gingipains limit the ability of target cells to bind TNF-α. Since both TNF receptors, TNFR I (p55) and TNFR II (p75), contain numerous arginine and lysine residues in their ectodomains (12 lysyl and 9 arginyl residues for p55; 6 lysyl and 13 arginyl residues for p75), it is possible that at least some of them are accessible to the enzymes. Therefore, the observed inhibition of TNF-α binding to the cells might result from gingipain-mediated degradation of TNF receptors. Confirmation of this hypothesis requires more-detailed studies.

Despite the ability of gingipains to degrade TNF-α, the cytokine has been found in GCF and tissue biopsy specimens from periodontal patients (19, 27). Inactivation of the TNF-α signaling pathway by gingipains may nonetheless occur in vivo. First, none of the studies compared the level of gingipain activity and the level of TNF-α in GCF samples. It is likely that the lack of detectable TNF-α in about 50% of the samples (52) is due to degradation of this cytokine. Second, although *P. gingivalis* is accepted as the main periodontopathogen, it is not always present in specific periodontal sites. Despite this fact, no correlation between the presence of the pathogen and the level of TNF-α in GCF has been determined. Finally, TNF-α production can occur in the tissue out of the operational range of gingipain activity, which is highest in the proximity of dental plaque infected with *P. gingivalis*.

In conclusion, we have demonstrated that gingipains, the bacterial cysteine proteinases elaborated by *P. gingivalis* are able not only to digest soluble TNF-α but also to degrade and inactivate the membrane form of the cytokine and to diminish the interactions of native TNF-α with the cells. This may result in significant attenuation of the host defense response, including the recruitment and activation of PMN at initial stages of infection. During the development of *P. gingivalis* infection, cytokines, such as IL-1, IL-6, RANKL, and, to a lesser extent, TNF-α produced beyond the area of high concentration of gingipains, may lead to the progression of periodontitis.

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REFERENCES

1. **Aggarwal, B. B.** 2003. Signalling pathways of the TNF superfamily: a double-edged sword. Nat. Rev. Immunol. **3:**745–756.
2. **Aggarwal, B. B., A. Samanta, and M. Feldmann.** 2001. TNF receptors, p. 1619–1632. *In* J. J. Oppenheim, M. Feldmann, S. K. Durum, T. Hirano, J. Vilcek, and N. A. Nicola (ed.), Cytokine reference, vol. 2. Academic Press, London, United Kingdom.
3. **Aggarwal, B. B., A. Samanta, and M. Feldmann.** 2001. TNF-α, p. 413–434. *In* S. K. Durum, T. Hirano, J. Vilcek, and N. A. Nicola (ed.), Cytokine reference, vol. 1. Academic Press, London, United Kingdom.
4. **Ally, N., J. C. Whisstock, M. Sieprawska-Lupa, J. Potempa, B. F. Le Bonniec, J. Travis, and R. N. Pike.** 2003. Characterization of the specificity of arginine-specific gingipains from *Porphyromonas gingivalis* reveals active site differences between different forms of the enzymes. Biochemistry **42:**11693–11700.
5. **Ausubel, F., R. Brent, R. Kingston, D. Moore, J. Seidman, J. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
6. **Azuma, Y., K. Kaji, R. Katogi, S. Takeshita, and A. Kudo.** 2000. Tumor necrosis factor-alpha induces differentiation of and bone resorption by osteoclasts. J. Biol. Chem. **275:**4858–4864.
7. **Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K. A. Schooley, M. Gerhart, R. Davis, J. N. Fitzner, R. S. Johnson, R. J. Paxton, C. J. March, and D. P. Cerretti.** 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature **385:**729–733.
8. **Bu, R., C. W. Borysenko, Y. Li, L. Cao, A. Sabokbar, and H. C. Blair.** 2003. Expression and function of TNF-family proteins and receptors in human osteoblasts. Bone **33:**760–770.
9. **Calkins, C. C., K. Platt, J. Potempa, and J. Travis.** 1998. Inactivation of tumor necrosis factor-alpha by proteinases (gingipains) from the periodontal pathogen, *Porphyromonas gingivalis*. Implications of immune evasion. J. Biol. Chem. **273:**6611–6614.
10. **Champagne, C. M., P. N. Madianos, S. Lieff, A. P. Murtha, J. D. Beck, and S. Offenbacher.** 2000. Periodontal medicine: emerging concepts in pregnancy outcomes. J. Int. Acad. Periodontol. **2:**9–13.
11. **Chen, Z., J. Potempa, A. Polanowski, M. Wikstrom, and J. Travis.** 1992. Purification and characterization of a 50-kDa cysteine proteinase (gingipain) from *Porphyromonas gingivalis*. J. Biol. Chem. **267:**18896–18901.
12. **Coligan, J., B. Dunn, H. Ploegh, D. Speicher, and P. Wingfield (ed.).** 1997. Current protocols in protein science. John Wiley & Sons, New York, N.Y.
13. **Curtis, M. A., H. K. Kuramitsu, M. Lantz, F. L. Macrina, K. Nakayama, J. Potempa, E. C. Reynolds, and J. Aduse-Opoku.** 1999. Molecular genetics and nomenclature of proteases of *Porphyromonas gingivalis*. J. Periodontal Res. **34:**464–472.
14. **Delima, A. J., S. Karatzas, S. Amar, and D. T. Graves.** 2002. Inflammation and tissue loss caused by periodontal pathogens is reduced by interleukin-1 antagonists. J. Infect. Dis. **186:**511–516.
15. **Drugarin, D., D. Onisei, A. Koreck, S. Negru, and M. Drugarin.** 1998. Proinflammatory cytokine production and PMN-elastase release from activated PMN cells in periodontal disease. Roum. Arch. Microbiol. Immunol. **57:**295–307.
16. **Duncan, L., M. Yoshioka, F. Chandad, and D. Grenier.** 2004. Loss of lipopolysaccharide receptor CD14 from the surface of human macrophage-like cells mediated by *Porphyromonas gingivalis* outer membrane vesicles. Microb. Pathog. **36:**319–325.
17. **Eissner, G., F. Kohlhuber, M. Grell, M. Ueffing, P. Scheurich, A. Hieke, G. Multhoff, G. W. Bornkamm, and E. Holler.** 1995. Critical involvement of transmembrane tumor necrosis factor-alpha in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin. Blood **86:**4184–4193.
18. **Genco, R., S. Offenbacher, and J. Beck.** 2002. Periodontal disease and cardiovascular disease: epidemiology and possible mechanisms. J. Am. Dent. Assoc. **133**(Suppl.)**:**14S–22S.
19. **Gorska, R., H. Gregorek, J. Kowalski, A. Laskus-Perendyk, M. Syczewska, and K. Madalinski.** 2003. Relationship between clinical parameters and cytokine profiles in inflamed gingival tissue and serum samples from patients with chronic periodontitis. J. Clin. Periodontol. **30:**1046–1052.
20. **Graves, D. T., and D. Cochran.** 2003. The contribution of interleukin-1 and tumor necrosis factor to periodontal tissue destruction. J. Periodontol. **74:**391–401.
21. **Graves, D. T., M. Oskoui, S. Volejnikova, G. Naguib, S. Cai, T. Desta, A. Kakouras, and Y. Jiang.** 2001. Tumor necrosis factor modulates fibroblast apoptosis, PMN recruitment, and osteoclast formation in response to *P. gingivalis* infection. J. Dent. Res. **80:**1875–1879.
22. **Grell, M., E. Douni, H. Wajant, M. Lohden, M. Clauss, B. Maxeiner, S. Georgopoulos, W. Lesslauer, G. Kollias, K. Pfizenmaier, et al.** 1995. The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. Cell **83:**793–802.
23. **Grell, M., G. Zimmermann, E. Gottfried, C. M. Chen, U. Grunwald, D. C.**

Huang, Y. H. Wu Lee, H. Durkop, H. Engelmann, P. Scheurich, H. Wajant, and A. Strasser. 1999. Induction of cell death by tumour necrosis factor (TNF) receptor 2, CD40 and CD30: a role for TNF-R1 activation by endogenous membrane-anchored TNF. EMBO J. **18:**3034–3043.

24. **Haas, E., M. Grell, H. Wajant, and P. Scheurich.** 1999. Continuous autotropic signaling by membrane-expressed tumor necrosis factor. J. Biol. Chem. **274:**18107–18112.
25. **Hajishengallis, G., H. Sojar, R. J. Genco, and E. DeNardin.** 2004. Intracellular signaling and cytokine induction upon interactions of *Porphyromonas gingivalis* fimbriae with pattern-recognition receptors. Immunol. Investig. **33:**157–172.
26. **Hehlgans, T., and D. N. Mannel.** 2002. The TNF-TNF receptor system. Biol. Chem. **383:**1581–1585.
27. **Hirose, K., E. Isogai, H. Miura, and I. Ueda.** 1997. Levels of *Porphyromonas gingivalis* fimbriae and inflammatory cytokines in gingival crevicular fluid from adult human subjects. Microbiol. Immunol. **41:**21–26.
28. **Hong, C. Y., S. K. Lin, S. H. Kok, S. J. Cheng, M. S. Lee, T. M. Wang, C. S. Chen, L. D. Lin, and J. S. Wang.** 2004. The role of lipopolysaccharide in infectious bone resorption of periapical lesion. J. Oral Pathol. Med. **33:**162–169.
29. **Jotwani, R., A. K. Palucka, M. Al-Quotub, M. Nouri-Shirazi, J. Kim, D. Bell, J. Banchereau, and C. W. Cutler.** 2001. Mature dendritic cells infiltrate the T cell-rich region of oral mucosa in chronic periodontitis: in situ, in vivo, and in vitro studies. J. Immunol. **167:**4693–4700.
30. **Kitamura, Y., S. Matono, Y. Aida, T. Hirofuji, and K. Maeda.** 2002. Gingipains in the culture supernatant of *Porphyromonas gingivalis* cleave CD4 and CD8 on human T cells. J. Periodontal Res. **37:**464–468.
31. **Lamont, R. J., and H. F. Jenkinson.** 1998. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. Microbiol. Mol. Biol. Rev. **62:**1244–1263.
32. **Lazdins, J. K., M. Grell, M. R. Walker, K. Woods-Cook, P. Scheurich, and K. Pfizenmaier.** 1997. Membrane tumor necrosis factor (TNF) induced cooperative signaling of TNFR60 and TNFR80 favors induction of cell death rather than virus production in HIV-infected T cells. J. Exp. Med. **185:**81–90.
33. **MacEwan, D. J.** 2002. TNF receptor subtype signalling: differences and cellular consequences. Cell. Signal. **14:**477–492.
34. **McNamara, K. M., S. E. Hall, R. S. Wilder, H. P. Lawrence, and S. Offenbacher.** 1999. Periodontitis and cytokine expression in CD14 deficient patients. J. Int. Acad. Periodontol. **1:**95–100.
35. **Mueller, C., N. Corazza, S. Trachsel-Loseth, H. P. Eugster, M. Buhler-Jungo, T. Brunner, and M. A. Imboden.** 1999. Noncleavable transmembrane mouse tumor necrosis factor-alpha (TNFα) mediates effects distinct from those of wild-type TNFα in vitro and in vivo. J. Biol. Chem. **274:**38112–38118.
36. **Okaji, M., H. Sakai, E. Sakai, M. Shibata, F. Hashimoto, Y. Kobayashi, N. Yoshida, K. Okamoto, K. Yamamoto, and Y. Kato.** 2003. The regulation of bone resorption in tooth formation and eruption processes in mouse alveolar crest devoid of cathepsin k. J. Pharmacol. Sci. **91:**285–294.
37. **Peters, J., and H. Baumgarten (ed.).** 1992. Monoclonal antibodies: a practical guide. Springer-Verlag, New York, N.Y.
38. **Pike, R., W. McGraw, J. Potempa, and J. Travis.** 1994. Lysine- and arginine-specific proteinases from *Porphyromonas gingivalis*. Isolation, characterization, and evidence for the existence of complexes with hemagglutinins. J. Biol. Chem. **269:**406–411.
39. **Potempa, J., A. Banbula, and J. Travis.** 2000. Role of bacterial proteinases in matrix destruction and modulation of host responses. Periodontol. 2000 **24:**153–192.
40. **Potempa, J., J. Mikolajczyk-Pawlinska, D. Brassell, D. Nelson, I. B. Thogersen, J. J. Enghild, and J. Travis.** 1998. Comparative properties of two cysteine proteinases (gingipains R), the products of two related but individual genes of *Porphyromonas gingivalis*. J. Biol. Chem. **273:**21648–21657.
41. **Potempa, J., R. Pike, and J. Travis.** 1997. Titration and mapping of the active site of cysteine proteinases from *Porphyromonas gingivalis* (gingipains) using peptidyl chloromethanes. Biol. Chem. **378:**223–230.
42. **Rifas, L.** 1999. Bone and cytokines: beyond IL-1, IL-6 and TNF-α. Calcif. Tissue Int. **64:**1–7.
43. **Schmid, E. F., K. Binder, M. Grell, P. Scheurich, and K. Pfizenmaier.** 1995. Both tumor necrosis factor receptors, TNFR60 and TNFR80, are involved in signaling endothelial tissue factor expression by juxtacrine tumor necrosis factor alpha. Blood **86:**1836–1841.
44. **Soga, Y., F. Nishimura, H. Ohyama, H. Maeda, S. Takashiba, and Y. Murayama.** 2003. Tumor necrosis factor-alpha gene (TNF-α) -1031/-863, -857 single-nucleotide polymorphisms (SNPs) are associated with severe adult periodontitis in Japanese. J. Clin. Periodontol. **30:**524–531.
45. **Stashenko, P., J. J. Jandinski, P. Fujiyoshi, J. Rynar, and S. S. Socransky.** 1991. Tissue levels of bone resorptive cytokines in periodontal disease. J. Periodontol. **62:**504–509.
46. **Sugawara, S., E. Nemoto, H. Tada, K. Miyake, T. Imamura, and H. Takada.** 2000. Proteolysis of human monocyte CD14 by cysteine proteinases (gingipains) from *Porphyromonas gingivalis* leading to lipopolysaccharide hyporesponsiveness. J. Immunol. **165:**411–418.
47. **Sundstrom, C., and K. Nilsson.** 1976. Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int. J. Cancer **17:**565–577.
48. **Suzuki, Y. J., M. Mizuno, and L. Packer.** 1994. Signal transduction for nuclear factor-κB activation. Proposed location of antioxidant-inhibitable step. J. Immunol. **153:**5008–5015.
49. **Tada, H., S. Sugawara, E. Nemoto, N. Takahashi, T. Imamura, J. Potempa, J. Travis, H. Shimauchi, and H. Takada.** 2002. Proteolysis of CD14 on human gingival fibroblasts by arginine-specific cysteine proteinases from *Porphyromonas gingivalis* leading to down-regulation of lipopolysaccharide-induced interleukin-8 production. Infect. Immun. **70:**3304–3307.
50. **Takashiba, S., K. Naruishi, and Y. Murayama.** 2003. Perspective of cytokine regulation for periodontal treatment: fibroblast biology. J. Periodontol. **74:**103–110.
51. **Weiss, T., M. Grell, B. Hessabi, S. Bourteele, G. Muller, P. Scheurich, and H. Wajant.** 1997. Enhancement of TNF receptor p60-mediated cytotoxicity by TNF receptor p80: requirement of the TNF receptor-associated factor-2 binding site. J. Immunol. **158:**2398–2404.
52. **Yavuzyilmaz, E., N. Yamalik, S. Bulut, S. Ozen, F. Ersoy, and U. Saatci.** 1995. The gingival crevicular fluid interleukin-1 beta and tumour necrosis factor-alpha levels in patients with rapidly progressive periodontitis. Aust. Dent. J. **40:**46–49.

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